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(54) Title: PSEUDOMONAS EXOTOXIN AS IMMUNOGENIC CARRIER IN SYNTHETIC CONJUGATE VACCINES

(57) Abstract

Pseudomonas exotoxin and variants thereof are potent immunogenic carrier proteins for GnRH. Vaccines containing a GnRH associated with Pseudomonas exotoxin are capable of eliciting high titres of anti-GnRH antibodies in animals, and are therefore useful for controlling fertility, reducing undesirable reproductive hormone driven behaviors, and treating sex steroid responsive tumors.

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TITLE OF THE INVENTION

PSEUDOMONAS EXOTOXIN AS IMMUNOGENIC CARRIER IN SYNTHETIC CONJUGATE VACCINES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is based on, and claims priority from, provisional application number 60/008,018 filed October 27, 1995.

BACKGROUND OF THE INVENTION

The present invention relates to a method for eliciting antibodies against gonadotropin releasing hormone (GnRH) in an animal comprising adminstering to said animal an immunogenic carrier system which comprises GnRH and Pseudomonas exotoxin or a variant thereof. The method of the present invention represents an effective and preferred method for sterilizing animals. The method may also be used to arrest development of steroid hormone stimulated tumors.

Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Sterilization may be used to control undesirable gonadal steroid hormone driven behavior such as aggression in males and estrus behavior in females, to improve feed efficiency and carcass quality in food animals such as swine and cattle, and to eliminate boar taint in the carcasses of male pigs.

Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermatic cord, retaining the testes in the inguinal ring, or use of a rubber band, placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes.

However, most of these "mechanical" castration methods have proven to be undesirable in one respect or another; for example they (1) are traumatic, (2) introduce the danger of anesthesia, (3) are apt to produce infection, and (4) require trained personnel. Moreover, all such mechanical castration methods result in complete abolition of the testes and this of course implies complete removal of the anabolic effects of any steroids which are produced by the testes and which act as stimuli to growth and protein deposition.

These drawbacks have caused consideration of various alternative sterilization techniques such as the use of chemical sterilization agents. One approach in chemical sterilization involves the use of a cytotoxic agent attached to a molecule that binds to GnRH receptors on gonadotrophs; upon the internalization of the GnRH-cytotoxin conjugate, the cytotoxic agent is released which kills the target cell.

The GnRH-cytotoxin approach is illustrated by the disclosure of WO90/09799, which teaches certain sterilizing agents comprising GnRH analogs coupled to a variety of toxins through an optional linking group consisting of 2-iminothiolane, SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate), bis-diazabenzidine and glutaraldehyde. WO93/15751 also discloses chimeric molecules of GnRH, or analogs thereof, and cytotoxins. The chimera of this disclosure is a molecule in which GnRH peptides are directly linked to a Pseudomonas exotoxin molecule. At each site of peptide binding to the toxin molecule, there is only one GnRH peptide bound. Administration of such chimeric molecules is reported to result in the destruction of GnRH receptor bearing cells in the pituitary gland, with concomitant reduction in the secretion of sex hormones. The utility of this approach will be determined by the rates of receptor endocytosis and intracellular processing in gonadotrophs. The ultimate result of this process is chemosterilization and reduction of steroid stimulated tumor proliferation.

UK Application No. 2,282,812 teaches GnRH attached to a cyclic scaffold containing multiple lysine units, termed a MAP (multiple

antigen peptide) or lysine tree, and the scaffold is in turn coupled to a cytotoxin such as Pseudomonas exotoxin. The use of the multi-lysine scaffold permits attaching more than one GnRH per cytotoxin linking site; however, the MAP approach is not necessarily an advantage because MAP conjugates generally have the attribute of high insolubility in hydrophobic and hydrophilic solvents rendering them more difficult to formulate.

Thus although GnRH coupled to Pseudomonas exotoxin has been previously reported, these conjugates were used to deliver the toxin into the cytosol of cells expressing GnRH receptors. The use of a GnRH-Pseudomonas exotoxin as a vaccine to raise anti-GnRH antibodies was not contemplated.

Another approach in animal sterilization involves the use of GnRH vaccines, i.e., immunosterilization. Typically a GnRH molecule, which is only weakly immunogenic, is coupled to an immunogenic macromolecule, such as a protein, in order to enhance the immunogenicity of GnRH; alternatively, a fusion protein containing a GnRH and an immunogenic peptide may be constructed for the same purpose. Animals administered the immunoconjugate or fusion protein develop antibodies against GnRH, which down regulate the action of GnRH resulting in the drastic reduction of sex hormones and the atrophy of androgen dependent organs. A number of GnRH immunoconjugates or fusion proteins suitable for use as vaccines have been described.

A commercial GnRH vaccine is currently being marketed in Australia by Arthur Webster & Co Pty Ltd under the name of Vaxstrate® for use in cattle (see e.g., R. M. Hoskinson et at, Aust. J. Biotechnol., 1990, 4:166). This vaccine, which is reported to consist of GnRH conjugated to ovalbumin, is poorly immunogenic. This vaccine is formulated in DEAE dextran and produces severe injection site reactions.

US 4,975,420 discloses immunosterilants comprising a GnRH analog in which the amino acid 1, 6 or 10 has been replaced by cysteine, coupled to a carrier protein.

WO88/05308 discloses immunoneutering compositions containing penta-, hexa-, or heptapeptide fragments of native GnRH conjugated with an immunogenic protein.

WO93/08290 describes fusion proteins comprising GnRH and a leukotoxin polypeptide. The leukotoxin serves as a carrier protein to increase the immunogenicity of the antigen.

EP 578,293 discloses fusion proteins comprising a part of an E.coli P-fimbrial filament and GnRH. This carrier system is said to be capable of eliciting a greatly improved immune response against GnRH, and when used in a vaccine, avoids the need for aggressive adjuvants such as complete/incomplete Freunds adjuvant (CFA/IFA).

WO92/19746 teaches recombinant polypeptides comprising GnRH, at least one T-cell epitope and a purification site.

WO90/02187 discloses fusion proteins comprising hepatitis B surface antigen and GnRH. The constructs are said to be sufficiently immunogenic to render unnecessary the use of adjuvants and multiple injections.

US 5,324,512 teaches GnRH linked through an N-terminal glutamine to a carrier protein. The conjugates are claimed to be useful as antifertility vaccine and in the treatment of prostate cancer.

WO94/25060 discloses immunogenic peptide containing GnRH, a T-cell epitope and, optionally, an invasin domain. The peptides are useful as antifertility vaccine and for treating androgen-dependent carcinoma.

UK 2,228,262 discloses conjugates in which [D-Lys^6]GnRH (i.e. amino acid 6 (glycine) of native GnRH has been replaced by D-Lys) is linked to a carrier protein through the ε-amino group of the D-Lys. The conjugates may be used to control fertility or for therapy of prostate cancer.

The vaccines as described generally require large amounts of the vaccine concomitant with severe adjuvants to obtain any antibody response. The adjuvants most commonly used are complete or incomplete Freund's adjuvant (CFA or IFA), which causes a variable amount of chronic inflammatory reaction at the site of injection, and are

generally not acceptable to regulatory authorities. These reported vaccines do not elicit adequate levels of anti-GnRH antibodies, and are incapable of providing 100% effectiveness in all animals of a given group.

As previously discussed, Pseudomonas exotoxin has been coupled to GnRH, and the resulting construct used for the destruction of gonadotrophs; the GnRH of the construct acts to deliver the toxin into cells bearing GnRH receptors, and once inside the cell, the toxin is released and exerts its cytotoxic activity to effect cell killing. The strategy of using a receptor binding ligand to deliver Pseudomonas toxin into the target cells has been well documented with a number of ligands other than GnRH.

Chaudhary, et al., PNAS USA 84:4538-4542 (1987) teach that hybrid fusion proteins formed between PE-40. a truncated variant of PE exotoxin A, and transforming growth factor-alpha and produced in bacteria using recombinant DNA techniques will bind to and kill human tumor cells possessing epidermal growth factor receptors.

Edwards, et al., Mol. Cell. Biol. 9: 2860-2867 (1989) describe the preparation of the modified TGF-alpha - PE-40 hybrid molecules that have been found to have utility in treating bladder tumor cells.

Heimbrook, et al., Proc. Natl. Acad. Sci. USA 87: 4697-4701 (1990) describe the in vivo efficacy of modified TGF-alpha - PE-40 in significantly prolonging the survival of mice containing human tumor cell xenografts.

U.S. patent 4,545,985 teaches that Pseudomonas exotoxin A can be chemically conjugated to an antibody or to epidermal growth factor. While this patent further teaches that these conjugates can be used to kill human tumor cells, these chemically linked toxins have been shown to have undesirable, nonspecific levels of activity.

Bailon, Biotechnology, pp. 1326-1329 Nov. (1988) teach that hybrid fusion proteins formed between PE-40 and interleukin 2 and produced in bacteria using recombinant DNA techniques will bind to and kill human cell lines possessing interleukin 2 receptors.

The use of Pseudomonas exotoxin to increase immunogenicity of a hapten is described in WO92/12173, which teaches fusion proteins of Pseudomonas exotoxin and specific regions of human P-glycoproteins; these fusion proteins are used to raise antibodies against P-glycoprotein. Conjugate vaccines composed of *Staphylococcus aureus* capsular polysaccharide and recombinant protein derived from Pseudomonas exotoxin A is reported in Fattom, A. et al, Inf. Immun., 1993, 61(3):1023-1032.

European patent application 0 261 671 teaches that a portion of the Pseudomonas exotoxin A protein can be produced which lacks the cellular binding function of the whole Pseudomonas exotoxin A protein but possesses the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein (mw 66,000). The portion of the Pseudomonas exotoxin A protein that retains the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein is called PE-40 (mw 40,000). PE-40 consists of amino acid residues 253-613 of the whole Pseudomonas exotoxin A protein as defined in Gray, et al., PNAS USA 81:2645-2649 1984. This patent application further teaches that PE-40 can be linked to transforming growth factor-alpha to form a hybrid fusion protein produced in bacteria using recombinant DNA techniques.

SUMMARY OF THE INVENTION

The present invention relates to the use of Pseudomonas exotoxin (PE) as an immunogenic carrier protein for GnRH. The GnRH-PE immunogenic carrier system may be GnRH-PE conjugates produced by chemical means, or GnRH-PE chimeric hybrid proteins produced using recombinant DNA technology. The GnRH-PE immunogenic carrier system may be used in mammalian or avian species for conditions in which it is desirable to reduce or eliminate reproduction, reproductive hormone driven behavior, physiology or anatomy, or to treat sex steroid responsive tumors.

DETAILED DESCRIPTION OF THE INVENTION

The present invention in one aspect provides a method for eliciting antibodies in a synthetic conjugate vaccine, and in particular anti-GnRH antibodies in an animal which comprises preferably administering to said animal an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereof, in an amount effective to elicit anti-GnRH antibodies. More particularly, there is provided a method for sterilizing an animal which comprises administering to said animal an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereof, in an amount effective to achieve sterilization.

The immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin may be a GnRH-Pseudomonas exotoxin conjugate wherein said GnRH is attached to the Pseudomonas exotoxin by chemical means via a linker, or it may be a GnRH-Pseudomonas exotoxin hybrid protein.

The following abbreviations are used:

CZE	capillary zonal electrophoresis
DCC	dicyclohexylcarbodiimide
DIEA	diisopropylethylamine
DMF	dimethylformamide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid, disodium salt
FMOC	9-fluorenylmethoxycarbonyl
HOBt	1-hydroxybenzotriazole
HPSEC	high performance size exclusion chromatography
MPS	3-maleimidopropionic acid N-hydroxysuccinimide ester
PE	Pseudomonas exotoxin
PyBOP	benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid

The term "GnRH", as used herein throughout the application, is intended to encompass the native GnRH and analogs or derivatives thereof that are capable of eliciting anti-GnRH antibodies when administered to a host in accordance with the present invention. When a particular GnRH molecule is meant, its amino acid sequence will be specified.

Native GnRH, also known as luteinizing hormone releasing hormone (LHRH), is a decapeptide having the amino acid sequence [SEQUENCE ID NO.: 1]:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

in which pGlu is pyroglutamate. Many analogs or derivatives of native GnRH have been reported, and they may be obtained by addition, deletion, replacement or other alterations of the native GnRH. Non-limiting examples of GnRH analogs or derivatives that may be suitable for use in the present inventions include those disclosed in UK Patent 2,228,262 (e.g. [D-Lys6]GnRH); US Patent 4,975,420 (e.g. [D-Cys^6]GnRH); US Patent 4,608,251 (e.g. N-terminus modified nonapeptide or decapeptide); European Patent Application 464,124 (e.g two GnRH in tandem); European Patent Application 293,530 (e.g. C-terminus extended GnRH); PCT Application 88/05308 (e.g. truncated fragments of GnRH); and US Patent 5,324,512 (pGlu of native GnRH replaced by Gln).

For use in chemically linked GnRH-PE conjugates, the native GnRH is preferably modified to include an amino acid that provides a functional group through which the GnRH can be linked to the Pseudomonas exotoxin; such an amino acid may be located at the N- or C-terminus, or it may replace amino acid 6 (Gly) of the native GnRH. More preferably, the GnRH includes a free amino or sulfhydryl group. Free amino group may be obtained by, for example, replacing the N-terminal pGlu with Gln, or by replacing Gly^6 of the native GnRH with Lys. Free sulfhydryl group may be obtained by replacing one of the

amino acids, for example, the amino acid at position 1, 6 or 10, with cysteine; or alternatively, a free amino group may be thiolated using homocysteine thiolactone or mercaptopropanoic acid to provide the free sulfhydryl group.

In a preferred embodiment for chemically linked conjugates, GnRH may be represented by the sequence [SEQUENCE ID NO.: 2]:

$$(B)_p\text{-Q-His-Trp-Ser-Tyr-W-T-Arg-U-V}$$

wherein:

B is	a thiol containing linker of the formula $HS\text{-}(CH_2)_n\text{-Co-Q-}$;
p is	0 or 1;
n is	1 to 10;
Q is	pGlu or Gln;
W is	a D- or L- amino acid selected from glycine, alanine, cysteine, homocysteine, ornithine, and lysine;
T is	Leu or Nle;
U is	Pro or 4-hydroxy-Pro; and
V is	$Gly\text{-}NH_2$, $D\text{-}Ala\text{-}NH_2$, NH-Et, NH-Pr or $Arg\text{-}Gly\text{-}NH_2$;

with the proviso that at least one of Q or W has a free amino or sulfhydryl group.

More preferred GnRH are of the formula [SEQUENCE ID NO.: 3]:

Q-His-Trp-Ser-Tyr-W-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

wherein Q and W are as defined above.

For hybrid proteins GnRH is preferably the native GnRH or an analog or derivative thereof that can be manufactured using recombinant DNA techniques, for example, two native GnRH molecules in tandem, or a GnRH analog comprising only natural amino acids.

Pseudomonas exotoxin is a protein (mw 66,000) composed of 613 amino acids arranged into 3 major, and one minor domain. The

preferred Pseudomonas exotoxins are variants thereof having decreased toxicity, for example, segments of Pseudomonas exotoxin wherein the binding or the ADP ribosylating activity has been attenuated or inactivated through deletion or partially deletion, insertion or substitution of amino acids in the binding or ribosylating domain, or where the PE holotoxin has been inactivated, for example by photoinactivation. The efficacy of PE as an immunogenic carrier protein is independent of the toxin activity of the PE; thus a PE-GnRH conjugate or hybrid protein may be inactivated, e.g. by photoinactivation, and still retain its immuogenic properties . One example of a Pseudomonas exotoxin with decreased toxin activity has had amino acids 1-252 deleted, which comprise most or all of the binding region and retaining amino acids 253-613 which contain the cell translocation region and the toxin region. This Pseudomonas exotoxin fragment has been identified as PE-40 - See Hwang et al., infra, Kondo et al J. Biol Chem 263 pg 9470-9475 (1988), Chaudhary et al, PNAS-USA, 87 pg 308-312 (1990) and US Patent 4892827 to Pastan et al.

The Pseudomonas exotoxin fragment PE-40 has been further modified by removing additional amino acids 365-380 to provide PE-38. PE-40 and PE-38 may be further modified by adding lysine containing oliogopeptide fragments to their N-termini. Addition of the 9 amino acid peptide ANLAEEAFK (the "Lys" peptide) to the N-terminus of PE-40 and PE-38 produces Pseudomonas exotoxins identified as Lys PE-40 and Lys PE-38, respectively; addition of the 10 amino acid peptide LQGTKLMAEE (the "NLys" peptide) produces Pseudomonas exotoxins identified as NLys PE-40 and NLys PE-38, respectively.

Replacing PE-38 lysines at 590 and 606 with glutamine, and lysine 613 with arginine generates the Pseudomonas exotoxin identified as PE-38QQR. Lys PE-38QQR and NLys PE-38QQR have, at their N-termini, the "Lys" and "NLys" peptides, respectively.

The various Pseudomonas exotoxin fragments are prepared using the techniques of biotechnology and recombinant DNA, and are described in Debinski and Pastan, Bioconjug. Chem., 1994, 5(1):40-46, and references cited therein.

The amino acid sequence of NLys PE-38QQR is shown below [SEQ ID No.: 4]; the underlined 4 amino acids represent the N-terminal amino acids of PE-38:

Met Ala Glu Gly....
Met Leu Gln Gly Thr Lys Leu Met Ala Glu Glu Gly Gly
Ser Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu
Pro Leu Glu Thr Phe Thr Arg His Arg Gln Pro Arg Gly
Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg
Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn
Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln
Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala
Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp
Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly Asp
Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe
Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly
Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His
Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly Tyr
His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe
Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala
Tyr Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly
Arg Ile Arg Asn Gly Ala Leu Leu Arg Val Tyr Val Pro
Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr Ser Leu Thr
Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr
Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu
Gly Trp Pro Leu Ala Glu Arg Thr Val Val Ile Pro Ser
Ala Ile Pro Thr Asp Pro Arg Asn Val Gly Gly Asp Leu
Asp Pro Ser Ser Ile Pro Asp Gln Glu Gln Ala Ile Ser
Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Gln Pro Pro
Arg Glu Asp Leu Arg

Other suitable Pseudomonas exotoxins include photoinactivated holotoxin and PE-38 (including Lys PE38, NLys PE-38, PE-38 QQR, Lys PE38QQR, and NLys PE-38QQR) in which the disulfide bond has been reduced.

The more preferred immunogenic carrier protein for the present invention is NLys PE-38QQR.

The immunogenic carrier system comprising a Pseudomonas exotoxin and a GnRH may be prepared by chemically coupling a GnRH to the PE carrier protein, or by using recombinant DNA techniques to produce GnRH-PE hybrid proteins. Each of these methods will be discussed hereinbelow.

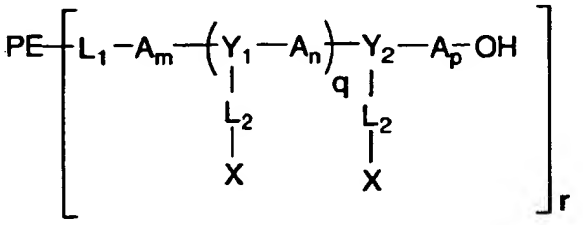
Methods for conjugating a peptide to a macromolecule is well known in the art, and are applicable to the preparation of GnRH-PE conjugates of the present invention. Generally, the peptide and the PE are linked through a cross linking reagent such as SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), glutaraldehyde, iminothiolane, N-acetyl-homocysteine thiolactone, bromoalkanoic anhydrides, maleimido-benzoyl-N-hydroxy-succinimide ester, 3-maleimidopropionic acid N-hydroxysuccinimide ester, and the like. Essentially any method where nucleophilic and electrophilic groups are provided on the reacting partners is sufficient to achieve linkage of peptides. The preferred cross linking agent for the present invention providing an electrophylic partner for the coupling reaction are active esters of maleimidoylalkanoic acids, and bromoalkanoic anhydrides. Preferred cross linking partners providing a nucleophile for the coupling reaction are N-acetyl homocysteine thiolactone and imino thiolactone.

In addition to the immunogenic carrier system wherein the GnRH is directly linked to the PE via a linker group, the present invention also comprehends a system wherein the GnRH component is first attached to an oligopeptidyl scaffold, and the GnRH-scaffold assembly is linked to the PE. Since the scaffold can be designed to contain more than one GnRH linking site, each binding site on the PE can carry more than one GnRH. The afore-mentioned cross linking reagents are also applicable for linking GnRH to the scaffold, and the scaffold to

the PE. In either type of conjugation, i.e. with or without the scaffold, any unconjugated haptens should be removed after conjugation reaction.

The term "GnRH-Pseudomonas conjugate" within the limits of the linker definition as described above thus encompasses any GnRH-PE conjugate with or without the scaffold.

The GnRH-scaffold PE conjugate may be represented by the formula:



I

wherein

A is independently an amino acid selected from Gly, Ser, Thr, β-Ala and Ala, with the proviso that at least one A is Ser or Thr;

L_1 is a linker optionally attached to an internal marker;

L_2 is independently a linker;.

X is a GnRH;

Y_1 and Y_2 are independently Lys or Orn;

m is 0 to 3;

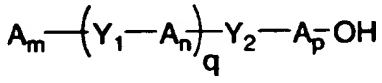
n is 1 to 10;

p is 0 to 1;

q is 1 or 2;

r is 1 to 10.

The term "scaffold" may be represented by the general formula shown below:



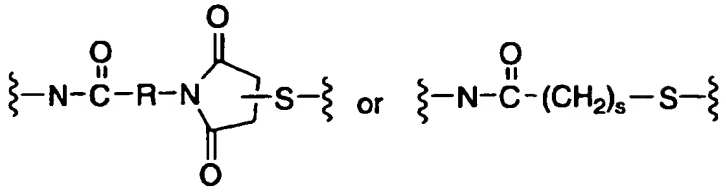
wherein the variables are as defined above.

The scaffold is a linear oligopeptide having up to a total of twenty seven amino acids in the sequence with at least two of those being independently ornithine or lysine. The other amino acids are selected from amino acids of small size with at least one of the non-Lys non-Orn amino acid being a hydrophilic amino acid. Examples of suitable small amino acids are alanine, β-alanine, glycine, serine, and threonine; serine and threonine are in addition hydrophilic. The hydrophilic amino acid is preferably serine or threonine, and up to 5 hydrophilic amino acid residues may be incorporated into the scaffold. More preferably, the scaffold oligopeptide has from 5 to 10 amino acids, one or two of which are Ser or Thr. The lysine/ornithine residues are separated from each other by at least one amino acid, preferably the spacer between lysine/ornithine residues is about 3 to 8 amino acids.

Examples of scaffold are Lys-A_n-Lys wherein n is 4 to 7, more preferably 5 or 6; one of A is Ser or Thr, and the others are selected from Gly, Ala and βAla. More specific examples include [SEQUENCE ID NO: 5] Lys-Gly-Gly-Ser-Gly-Gly-Lys and [SEQUENCE ID NO: 6] Lys-Gly-Gly-Thr-Gly-βAla-Gly-Lys.

The term "internal marker" means an unnatural amino acid that has been incorporated to facilitate characterization of the conjugate and the calculation of the ratio of GnRH to carrier protein. Examples include β-Ala and norleucine.

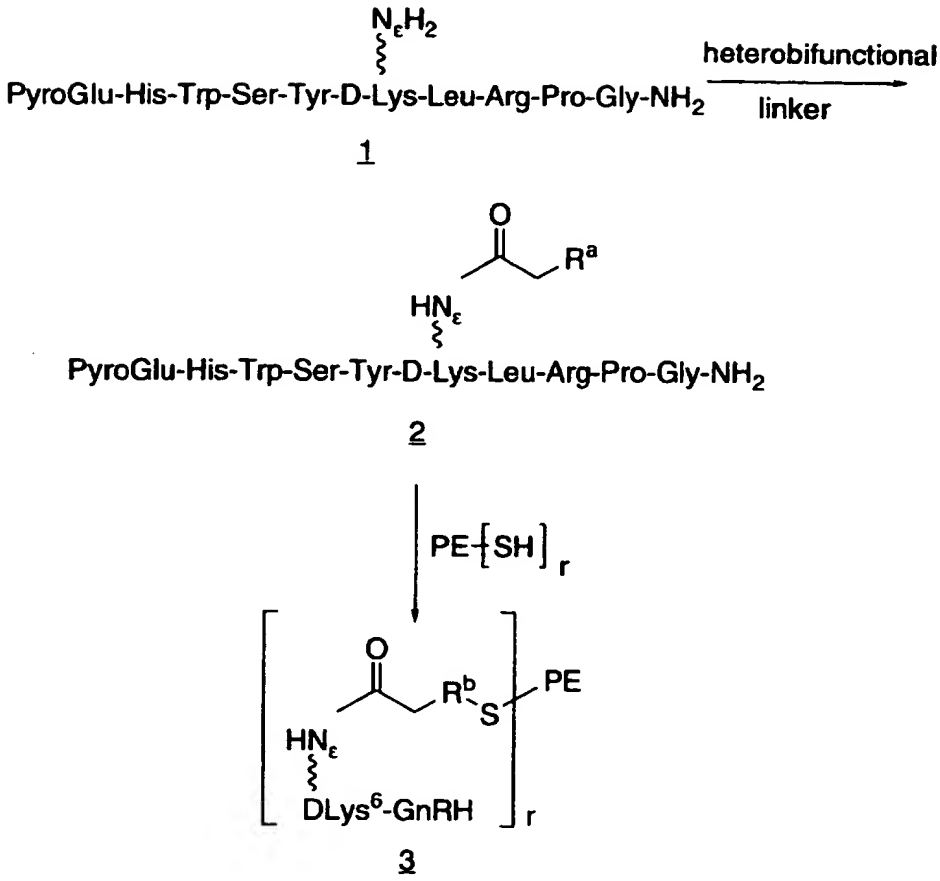
The "linker" for chemical conjugation of GnRH and Pseudomonas exotoxin may be derived from any heterobifunctional cross-linking agents carrying functionalities that are reactive with amino group and sulfhydryl group; for examples, SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), glutaraldehyde, iminothiolane, bromoalkanoic anhydrides, maleimido-benzoyl-N-succinimide ester, 3-maleimidopropionic acid N-hydroxysuccinimide ester, and the like may be used. Preferred linkers have the formula (where the terminal N and S are derived from the peptides that are linked):



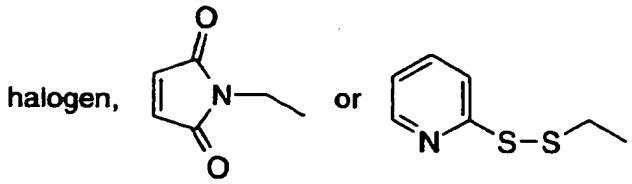
wherein R is C_1-C_5 alkylene, phenyl or C_5-C_6 cycloalkylene; and s is 1 or 2.

The two strategies for constructing chemically conjugated immunogenic carrier system, i.e. a GnRH linked to PE via a linker, and GnRH-scaffold linked to PE, are exemplified in the following schemes.

SCHEME 1



In Reaction Scheme 1 R^a is a linker group, such as



, and R^b is correspondingly a bond, succinimidylmethyl, or thiomethyl; r is 1-10.

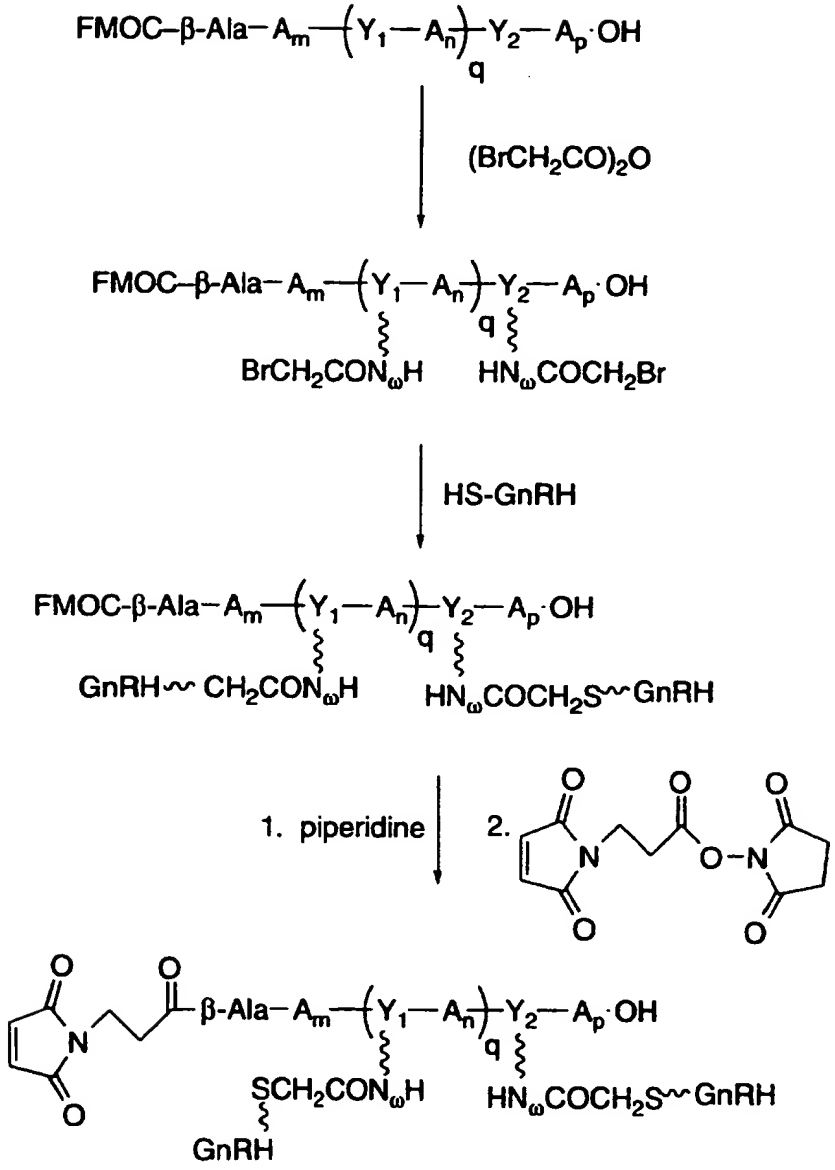
The first step is the reaction of DLys^6-GnRH with a heterobifunctional linker such as maleimidopropanoyl N-hydroxysuccinimide (MPS), bromoacetic anhydride, or SPDP. The

DLys^6-GnRH is prepared using known peptide synthesis techniques, preferably solid phase peptide synthesis. "$N_\varepsilon H_2$" is the epsilon amino group of lysine.

The reaction is carried out in a polar solvent with a base selected from either (a) a non-nucleophilic organic base such as N,N-diisopropyl ethylamine (DIEA) or (b) a weak inorganic base such as sodium or potassium carbonate. The polar solvent can be N,N-dimethylformamide, water, acetonitrile or mixture thereof. N,N-Dimethylformamide is preferred. The reaction is carried out at from 0 to 25°C, preferably at room temperature and is generally complete in from 10 to 90 minutes. The work-up of the reaction is to initially neutralize the base present with an acid such as trifluoroacetic acid, and the pH of the mixture is brought to about 2-4. The product is than isolated using techniques known to those skilled in the art.

The coupling of the linker-DLys^6-GnRH 2 with the Pseudomonas exotoxin having one or more free sulfhydryl functionality is carried out under nitrogen at a pH of from 8 to 10 with a excess of the linker-DLys^{6}GnRH. Generally from 2 to 20 equivalents of the GnRH reagent are used for each equivalent of the thiol substituent on the Pseudomonas exotoxin. The reaction is generally very fast and is complete in just 1-5 minutes although further aging of up to 2 hours has not been found to be detrimental. The DLys^6-GnRH conjugate is isolated using techniques known to those skilled in the art. It has been found that dialysis of the reaction mixture is a convenient method for the removal of unwanted products. Since the conjugated product will generally be administered by injection, the resultant dialysis solution may be sterile filtered and used directly for percutaneous administration.

SCHEME 2

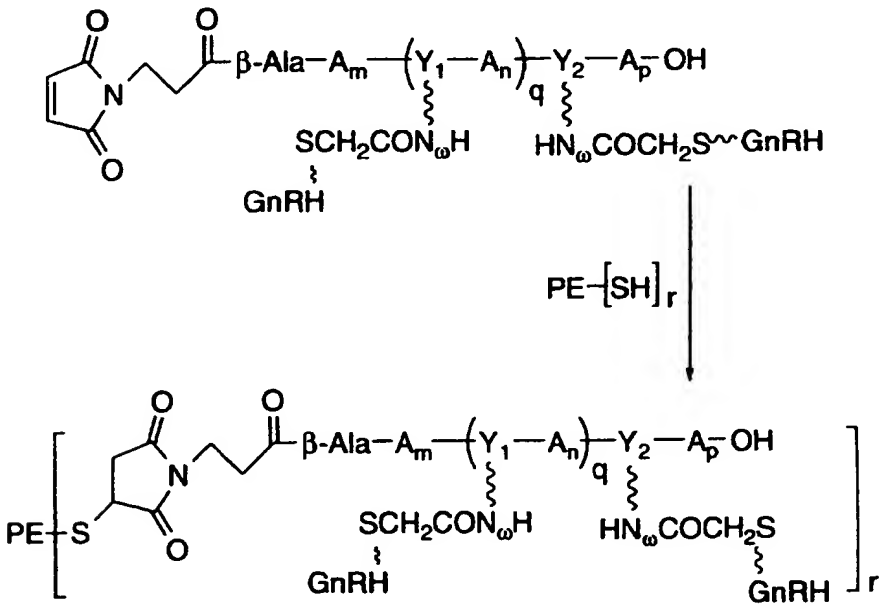


wherein the variables are as defined above.

In Scheme 2, the scaffold bearing a protected internal marker β-alanine is reacted with bromoacetic anhydride to effect bromoacetylation of the terminal amino groups (N_ω) of the Y_1 and Y_2

residues of the scaffold. The reaction is carried out under nitrogen in an inert organic solvent such as methylene chloride, dimethylformamide or a combination thereof, typically at ambient temperature. The bromoacetylated scaffold is reacted with a GnRH having a free sulfhydryl group, for example [DCys^6]GnRH or HSCH$_2$CH$_2$CO-[Gln^1]GnRH, resulting in scaffold supported GnRH. The reaction is carried out in aqueous acetonitrile at room temperature, and the pH of the reaction mixture is preferably maintained at about 8, and generally between 7.5 and 8.5. The Fmoc protecting group is removed using e.g., piperidine, and the deprotected peptide is reacted with a maleimidyl alkanoic acid activated ester such as MPS, at room temperature in an inert organic solvent such as dimethylformamide, and in the presence of a base such as diisopropylethylamine, to provide maleimidated scaffold supported GnRH.

SCHEME 3



In Scheme 3, the Pseudomonas exotoxin carrier protein having a free thiol is reacted with the scaffold-supported GnRH product

shown in Scheme 1 to provide the desired conjugate. The free thiol may be associated with a free cysteine, or generated by reducing disulfide bond(s) in the carrier protein using, for example, dithiothreitol, or introduced onto the carrier protein by reacting the carrier protein with a thiolating agent, such as N-alkanoylhomocysteine thiolactone. More than one free thiol may be present on the carrier protein, and therefore more than one scaffold-supported GnRH may be loaded onto the carrier protein.

It will be understood that the reaction sequences in the above schemes only illustrate the invention, and can be adapted or modified by one of ordinary skill in the art without undue experimentation to arrive at other variations within the scope of the invention.

The immunogenic carrier system of the present invention may also be hybrid proteins of GnRH and Pseudomonas exotoxin A . Such hybrid proteins contain contiguous sequences of the constituent proteins or peptides. The hybrid proteins are preferably manufactured through expression of recombinant DNA sequences.

The DNAs used in the practice of the invention may be natural or synthetic. The recombinant DNA segments containing the nucleotide sequences coding for the embodiments of the present invention can be prepared by the following general processes:

(a) A desired DNA sequence is cut out from a plasmid in which it has been cloned, or the sequence can be chemically synthesized;

(b) Then a second DNA sequence, the targeted DNA sequence, is cleaved at a specific location; and

(c) The desired DNA sequence is then brought into alignment with the cut in the targeted DNA sequence and the two sequences are connected together through standard ligation procedures. The resulting recombinant gene is ligated down stream from a suitable promoter in an expression vector.

Techniques for cleaving and ligating DNA as used in the invention are generally well known to those of ordinary skill in the art and are described in Molecular Cloning, A Laboratory Manual, (1989) Sambrook, J., et al, Cold Spring Harbor Laboratory Press.

As the promoter used in the present invention, any promoter is usable as long as the promoter is suitable for expression in the host used for the gene expression. The promoters can be prepared enzymatically from the corresponding genes, or can be chemically synthesized.

Conditions for usage of all restriction enzymes are in accordance with those of the manufacturer, including instructions as to buffers and temperatures. The enzymes may be obtained from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim and Promega.

Ligations of vector and insert DNAs are performed with T4 DNA ligase in 66 mM Tris-HCl, 5 mM MgCl2, 1mM DTE, 1mM ATP, pH 7.5 at 15°C for up to 24 hours. In general 1 to 200 ng of vector and 3-5x excess of insert DNA are preferred.

Selection of E. coli containing recombinant plasmids involve streaking the bacteria onto appropriate antibiotic containing LB agar plates or culturing in shaker flasks in LB liquid (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.4) containing the appropriate antibiotic for selection when required. Choice of antibiotic for selection is determined by the resistance markers present on a given plasmid or vector. Preferably, vectors are selected by ampicillin.

Culturing E. coli involves growing in Erlenmeyer flasks in LB supplemented with the appropriate antibiotic for selection in an incubation shaker at 250-300 rpm and 37°C. Other temperature fro 25 - 37 C could be utilized. When cells are grown for protein production, they are induced at A560 = 1 with IPTG to a final concentration of 0.4 mM. Other cell densities in log phase growth can alternatively be chosen for induction.

Harvesting involves recovery of E. coli cells by centrifugation. For protein production, cells are harvested 3 hours after induction though other times of harvesting could be chosen.

In the present invention, any vector, such as a plasmid, may be used as long as it can be replicated in a prokaryotic or eukaryotic cell as a host.

The host cell of choice is E. coli HB101. However, a number of other E. coli strains would be suitable. For routine cloning, E. coli strain DH5a(BRL) can be used.

In the present invention, the hybrid proteins can be separated and purified by appropriate combinations of well-known separating and purifying methods. These methods include methods utilizing a solubility of differential such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high pressure liquid chromatography, methods utilizing a difference in isoelectric point, such as isoelectricfocusing electrophoresis, and methods using denaturation and reduction and renaturation and oxidation.

Immunogenic carrier system of the present invention have utility in human medicine as well as in veterinary medicine. This follows from the fact that there are several important biological reasons for employing castration and antifertility drugs in humans. For example, breast and prostate cancers are but two examples of sex steroid-dependent tumors which respond to such hormonal manipulation. Another area of application in human medicine is treatment of endometriosis. This condition, which produces painful growth of endometrial tissue in the female peritoneum and pelvis, also responds to inhibition of sex steroid synthesis. Those skilled in this art will also appreciate that the herein disclosed compounds could be used to partially reduce sex-steroid secretions, and thus reduce or eliminate certain hormone related effects in certain disease states.

The immunogenic carrier system of the present invention can also be used in veterinary medicine or animal husbandry for conditions in which it is desirable to reduce or eliminate reproduction and/or reproductive hormone driven behavior, physiology or anatomy. Sterilization of animals has primarily been achieved by surgical removal

of the gonads. Surgery necessarily involves some degree of pain, trauma and stress for the animal with the potential for infection and death. In food animals, neutering has been used as a means of controlling undesirable behaviors or meat characteristics but it has resulted in substantial production losses. In the case of swine, intact males have a much higher feed efficiency (approximately 18%) than castrates. However, androstenone is deposited in the fat of the intact male giving the meat an undesirable smell and odor.

Accordingly, another aspect of the present invention provides a method of sterilizing animals comprising administering to said animal an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereo, in an amount effective to achieve sterilization. Vaccination using the immunogenic carrier system of the present invention eliminates the need for surgical neutering. Therefore, it eliminates the pain, stress, trauma, infection, death, production loss and animal welfare issues associated with surgical neutering. Desirable sequellae of vaccination include transient sterilization, controlling undesirable gonadal steroid hormone driven behavior such as aggression in males and estrus behavior in females, improving feed efficiency and carcass quality in food animals such as swine and cattle and a method for eliminating boar taint in the carcasses of male pigs.

The dose/time adjustments associated with the use of these compounds can vary considerably and will depend on a variety of factors such as the species of animal to be treated, the particular GnRH and/or carrier used, the adjuvant, the age of the animal, and the desired outcome of vaccination. In general, the immunogenic carrier system are administered by subcutaneous or intramuscular injection into a mammal at a rate of 1 μg to 1000 μg of conjugate per dose . A single dose of the immunogenic carrier system of the present invention may be all that is required to achieve sterilization, but multiple doses spaced at one to six week intervals are alternative sterilization schemes. Furthermore, as sterilization agents, the compounds of this invention can be used before or after puberty; thus they can delay sterilization, which is especially

useful in those areas of animal husbandry where the anabolic benefits associated with the flexibility of timing of non-surgical sterilization can contribute positively to feed efficiency, meat production and/or quality.

In swine, the immunogenic carrier system can be used to maximize the boar-like growth efficiency and carcass quality while eliminating the offensive odor and taste of boar meat. This can be accomplished with one or two intramuscular or subcutaneous injections administered at various times during the grow out period. An example of convenient and efficacious schedule consists of an initial vaccination at the time of housing in the grower/finisher facility (9-16 weeks of age) with a booster late in the grow out (between 18 and 22 weeks of age). Each vaccination may be at a dose of about 1 μg to about 1000 μg of the immunogenic carrier system, preferably about 10 μg to about 100 μg is used. For a single dose regimen, the amount of the immunogenic carrier system is generally at a higher level than that used for two or more doses.

Feedlot cattle could be treated in a manner similar to that used for swine, with vaccinations at the time of entry into the feedlot and at another time which would be determined by the effect desired, i.e., prevention of pregnancy in the females or growth maximization in the males. Females need to complete the vaccination prior to entering mixed sex housing to prevent pregnancy; however, in housing segregated by sex, vaccination could occur at time of arrival and then 4-12 weeks later to prevent estrus. Bulls should be vaccinated late enough to maximize feed efficiency, but early enough to prevent aggression and to provide marbling of meat.

In companion animals such as dogs and cats, the GnRH vaccine could be administered by subcutaneous or intramuscular injection at times when the standard vaccinations are given (between 6 and 21 weeks of age with a booster at 6 months and annual boosters thereafter).

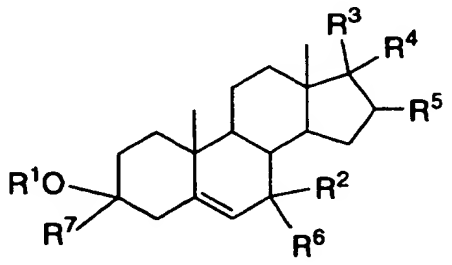
For neutering adult animals such as dogs, cats and horses, two doses administered at 2-8 week intervals followed by and annual boosters should be sufficient to produce neutering. The actual dose and formulation remains to be determined and may vary with the particular immunogenic carrier system used. However, a dose of 1 to 2000 ug,

preferably about 500 ug of an immunogenic carrier system of the present invention formulated on alum and administered in a volume of 1-3 mls may be sufficiently potent when administered as described above.

In man, immunogenic carrier system of the present invention can be used to treat sex steroid responsive tumors. Two doses at 1 to 1000 ug per dose of the vaccine can be administered at 2 to 8 week intervals with boosters at 6 to 12 months until the tumor is eliminated or ceases to be responsive to hormonal therapy.

The immunogenic carrier system of the present invention can be used for the above-mentioned application without the use of aggressive adjuvants such as Complete Freund's Adjuvant, which cause injection site lesions and downgrading of meat animal carcasses. Suitable adjuvants are any of those substances recognized by the art as enhancing the immunological response of a mammal to an immunogen without causing an unacceptable adverse reaction, and include aluminum compounds such as $Al(OH)_3$, $AlPO_4$, $Al_2(SO_4)_3$, water-in-oil emulsions such as Incomplete Freund's Adjuvant (IFA), Bayol F® or Marcol F®, vitamin-E acetate solubilisate, saponins, muramyl dipeptides in an appropriate solvent such as squalane or squalene.

In a preferred embodiment, the immune carrier system of the present invention is administered in an oil-in-water emulsion containing a metabolizable oil, a non-ionic surfactant such as a polyoxypropylene (POP)-polyoxyethylene (POE) block polymer, an emulsifier, and optionally an immune response enhancer of formula 1



1

wherein

R^1 is H, C_{2-8} alkenyl, C_{1-8} alkyl, benzyl, phenyl or COR^4, wherein R^4 is H, C_{1-8} alkyl, C_{2-8} alkenyl, benzyl or phenyl wherein the phenyl moiety may have up to three substituents selected from the group consisting of hydroxy, carboxy of 1-4 carbon atoms, halo, C_{1-4} alkoxy, C_{1-4} alky, and C_{2-4}alkenyl, SO_3M or PO_3M, wherein M is H or sodium or potassium;

R^2 is H or OR^1;

R^3 is OR^1 or R^3 and R4 together form an oxo;

R^4, R^5, R^6, and R^7 are independently H or methyl;

with the proviso that when R^3 and R4 together form an oxo, R^5, R^6, R^7 and R^2 are each H; and that when R^2 is H, R^4, R^5, R^6 and R^7 are each hydrogen, and R^3 is OR^1.

In the vaccine composition, the metabolizable oil may be an oil of 6 to 30 carbon atoms including alkanes, alkenes, alkynes, and their corresponding acids and alcohols, the ethers and esters therof, and mixtures thereof. The oil may be any vegetable oil, fish oil, animal oil or synthetically prepared oil which can be metabolized in the body of the subject to which the adjuvant is administered, and which is not toxic to the organism. Examples of vegetable oil include peanut oil, soybean oil, coconut oil, olive oil, safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil, and corn oil. Animal oils are usually solids at physiological temperature; however, fatty acids are obtainable from animal fats by partial or complete triglyceride saponifiction which provides the free fatty acids. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil and shark liver oils, exemplify several of the fish oils. Whale oils such as spermaceti are also acceptable. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene. Squalane, the saturated analog of squalene is a particularly preferred oil for the present invention. The oil component of the adjuvant compositions and vaccines of the invention will usually be

present in an amount between 1% and 10%, but preferably in an amount between 2.5 and 5%.

The term "polyoxypropylene-polyoxyethylene block polymer" refers to a polymer made by the sequential addition of propylene oxide and then ethylene oxide to a low molecular weight, reactive compound, usually propylene glycol. These block polymers can be prepared by the methods set out in U.S. Pat. No. 2,674,619 issued to Lunsted, and are commercially available from BASF-Wyandotte under the trademark Pluronic®. The characteristics of these block polymers are determined by the molecular weight of the POP nucleus and of the percentage POE in the product. The POP section imparts hydrophobic characteristics to the block polymer, while the POE section imparts hydrophilic characteristics.

Pluronic® block polymers are designated by a letter prefix followed by a two or a three digit number. The letter prefixes (L, P, or F) refer to the physical form of each polymer, (liquid, paste, or flakeable solid). The first one or two digits is a code for the average molecular weight of the POP base, while the last digit indicates the amount of POE. For example, Pluronic® L101 is a liquid having a polyoxypropylene base of average molecular weight 3,250, with 10% polyoxyethylene present at the ends of the molecule. The preferred block polymers are those which are liquid over a temperature range between about 15°-40° C. In addition, polymer mixtures of liquid and paste, liquid, paste and flakeable solid or liquid and flakeable solid mixtures which are liquid within the specified temperature range may have utility in this invention.

Preferred block polymers are those having a POP base ranging in molecular weight between about 2250 and 4300 and POE in an amount between about 1 and 30%. More preferred are those polymers wherein POP has a molecular weight falling between 3250 and 4000 and the POE component comprises 10-20%. The Pluronic® block polymers L101, L121 and L122 fall within this definition. Most preferred are the block polymers wherein POP has a molecular weight of 4000 and POE in an amount of 10% or POP has a molecular weight of 3250 and POE in an amount of 10% e.g. Pluronic® block polymers L121 and L101

respectively. The block polymer is preferably used in an amount between 0.001 and 10%, most preferably in an amount between 0.001 and 5%.

The term "emulsifier" refers to non-toxic surface active agents capable of stabilizing the emulsion. There are a substantial number of emulsifying and suspending agents generally used in the pharmaceutical sciences. These include naturally derived materials such as gums, vegetable protein, alginates, cellulose derivatives, phospholipids (whether natural or synthetic), and the like. Certain polymers having a hydrophilic substituent on the polymer backbone have emulsifying activity, for example, povidone, polyvinyl alcohol, and glycol ether-based compounds. Compounds derived from long chain fatty acids are a third substantial group of emulsifying and suspending agents usable in this invention. Though any of the foregoing emulsifiers can be used so long as they are non-toxic, glycol ether-based emulsifiers are preferred. Preferred emulsifiers are non-ionic. These include polyethylene glycols (especially PEG 200, 300, 400, 600 and 900), Span®, Arlacel®, Tween®, Myrj®, Brij® (all available from ICI America Inc., Wilmington, Del.), polyoxyethylene, polyol fatty acid esters, polyoxyethylene ether, polyoxypropylene fatty ethers, bee's wax derivatives containing polyoxyethylene, polyoxyethylene lanolin derivatives, polyoxyethylene fatty glycerides, glycerol fatty acid esters or other polyoxyethylene acid alcohol or ether derivatives of long-chain fatty acids of 12-21 carbon atoms. The presently preferred emulsifier is Tween® 80 (otherwise known as polysorbate 80 or polyoxyethylene 20 sorbitan monooleate), although it should be understood that any of the above-mentioned emulsifiers would be suitable after lack of toxicity is demonstrated. The emulsifier is usually used in an amount of about 0.05 to about 0.5%, preferably about 0.2 to 1%.

The aqueous portion of the adjuvant compositions of the invention is preferably buffered isoosmotic saline. It is preferred to formulate these solutions so that the tonicity is essentially the same as normal physiological fluids in order to prevent post-administration swelling or rapid absorption of the composition due to differential ion concentrations between the composition and physiological fluids. It is

also preferred to buffer the saline in order to maintain a pH compatible with normal physiological conditions. Also, in certain instances, it may be necessary to maintain the pH at a particular level in order to insure the stability of certain composition components, such as the glycopeptides. Any physiologically acceptable buffer may be used herein, but it has been found that it is most convenient to use a phosphate buffer. Any other acceptable buffer such as acetate, Tris, bicarbonate, carbonate, and the like can be used as a substitute for a phosphate buffer. It is preferred to use phosphate buffered saline, or saline buffered with a mixture of phosphate and acetate.

The immune response enhancers of formula 1, are either compounds well known in the art (e.g. dehydroepiandrosterone) or they may be prepared according to the disclosures of US Patent 5,277,907 of R. M. Loria, or WO95/10527 of Neurocrine Biosciences. In a preferred embodiment of the vaccine composition, an immunopotentiating amount of the immune response enhancer is included. More preferably, the immune response enhancer is a compound of formula a wherein R^1, R^2, R^5, R^6, and R^7 are each H, and R^3 and R^4 together form an oxo group, this compound being dehydroepiandrosterone or DHEA.

In the vaccine composition of the present invention the immunogenic carrier system may be any GnRH-PE conjugates or GnRH-PE hybrid The immunogenic carrier system of the present invention may also be hybrid proteins of GnRH and Pseudomonas exotoxin A . Such hybrid proteins contain contiguous sequences of the constituent proteins/peptides. The hybrid proteins are preferably manufactured through expression of recombinant DNA sequences.

The DNAs used in the practice of the invention may be natural or synthetic. The recombinant DNA segments containing the nucleotide sequences coding for the embodiments of the present invention can be prepared by the following general processes:

(a) A desired DNA sequence is cut out from a plasmid in which it has been cloned, or the sequence can be chemically synthesized;

(b) Then a second DNA sequence, the targeted DNA sequence, is cleaved at a specific location; and

(c) The desired DNA sequence is then brought into alignment with the cut in the targeted DNA sequence and the two sequences are connected together through standard ligation procedures. The resulting recombinant gene is ligated down stream from a suitable promoter in an expression vector.

Techniques for cleaving and ligating DNA as used in the invention are generally well known to those of ordinary skill in the art and are described in Molecular Cloning, A Laboratory Manual, (1989) Sambrook, J., et al, Cold Spring Harbor Laboratory Press.

As the promoter used in the present invention, any promoter is usable as long as the promoter is suitable for expression in the host used for the gene expression. The promoters can be prepared enzymatically from the corresponding genes, or can be chemically synthesized.

Conditions for usage of all restriction enzymes are in accordance with those of the manufacturer, including instructions as to buffers and temperatures. The enzymes may be obtained from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim and Promega.

Ligations of vector and insert DNAs are performed with T4 DNA ligase in 66 mM Tris-HCl, 5 mM MgCl2, 1mM DTE, 1mM ATP, pH 7.5 at 15°C for up to 24 hours. In general 1 to 200 ng of vector and 3-5x excess of insert DNA are preferred.

Selection of E. coli containing recombinant plasmids involve streaking the bacteria onto appropriate antibiotic containing LB agar plates or culturing in shaker flasks in LB liquid (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.4) containing the appropriate antibiotic for selection when required. Choice of antibiotic for selection is determined by the resistance markers present on a given plasmid or vector. Preferably, vectors are selected by ampicillin.

Culturing E. coli involves growing in Erlenmeyer flasks in LB supplemented with the appropriate antibiotic for selection in an incubation shaker at 250-300 rpm and 37°C. Other temperature fro 25 - 37 C could be utilized. When cells are grown for protein production, they

are induced at A560 = 1 with IPTG to a final concentration of 0.4 mM. Other cell densities in log phase growth can alternatively be chosen for induction.

Harvesting involves recovery of E. coli cells by centrifugation. For protein production, cells are harvested 3 hours after induction though other times of harvesting could be chosen.

In the present invention, any vector, such as a plasmid, may be used as long as it can be replicated in a prokaryotic or eukaryotic cell as a host.

The host cell of choice is E. coli HB101. However, a number of other E. coli strains would be suitable. For routine cloning, E. coli strain DH5a(BRL) can be used.

In the present invention, the hybrid proteins can be separated and purified by appropriate combinations of well-known separating and purifying methods. These methods include methods utilizing a solubility of differential such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse-phase high pressure liquid chromatography, methods utilizing a difference in isoelectric point, such as isoelectricfocusing electrophoresis, and methods using denaturation and reduction and renaturation and oxidation.

proteins capable of eliciting anti-GnRH antibodies in the animal given the vaccine. Preferably, GnRH-PE conjugates are used, and these may include the scaffold supported GnRH-PE conjugates, as well as other known GnRH-PE conjugates.

In a preferred embodiment of the vaccine composition, the oil-in-water emulsion comprises squalane, Tween 80 and Pluronic L121. More preferred, the vaccine includes DHEA as the immune response

enchancer. Even more preferably, the Pseudomonas exotoxin is NLys PE38QQR.

The oil-in-water emulsion adjuvant composition is prepared by emulsification using a mixer to form a homogenous emulsion. Typically, the adjuvant composition is microfluidized prior to adding the GnRH-conjugate; the emulsion is cycled through the microfluidizer about 2-20 times. The GnRH-conjugate is then mixed with the adjuvant composition, and the mixture may be again cycled through the microfluidizer. The immune response enhancer, e.g. DHEA, if included in the vaccine composition, may be added to the adjuvant composition prior to microfluidization, or it may be added after the GnRH-conjugate has been mixed with the adjuvant composition. In the latter case, the entire mixture should be microfluidized again, generally 2-10 times through the microfluidizer. The oily particles in the emulsion preferably have diameters of about 0.03 μm and 0.5 μm, more preferably between 0.05 and 0.2 μm.

PREPARATION OF GNRH

Preparation 1. DLys^6-GnRH : [SEQUENCE ID NO: 7] pGlu-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (SPPS) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (2 h, RT) from the resin using reagent R (1 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC (Waters PrepPak®25 x 10™ C_{18}; 10 mL/min; 10-20% B, 0-20 min.; then 20-35% B, 20-40 min.; λ=230 nm).

DLys^6-GnRH: FAB-MS (positive ion, NBA matrix) Calc. M+1 1254.44; Found M+1 = 1254.4

Preparation 2. [DCys^6]GnRH : [SEQUENCE ID NO: 8] pGlu-His-Trp-Ser-Tyr-DCys-Leu-Arg-Pro-Gly-NH_2

Rink amide resin (521 mg) was coupled sequentially with Gly, Pro, Arg, Leu, D-Cys, Tyr, Ser, Trp, His, Pyroglutamic acid. The Arg and Gly residues were double-coupled and the other residues were single-coupled. The resin was washed with methanol three times and dried under nitrogen (1.069 g resin = 0.539 g weight gain).

The peptide was cleaved for three hours, the resin was filtered off, and the peptide was dried under vacuum. The residue was triturated with diethyl ether and the precipitated peptide was collected by suction filtration and lyophilized (431.7 mg).

A portion of the peptide (195.5 mg) was dissolved in 0.1% TFA, 10% acetonitrile (3 mL), filtered, and purified by RPHPLC (15-45% acetonitrile over 30 minutes using two 25X10 RCM delta pak C18 columns in tandem. A repeat purification of a second portion of peptide was conducted (190.5 mg). Peak fractions were collected, combined and lyophilized, and an aliquot was analyzed by FAB-MS and amino acid analysis. The predicted mass (1229) and amino acid composition were confirmed.

Preparation 3. 3-(Mercaptopropanoyl)-[Gln^1]GnRH : [SEQUENCE ID NO: 9] $HSCH_2CH_2CONH$-Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

The peptide was prepared, up to the Gln^1 residue, on an ABI 431A peptide synthesizer (Fmoc chemsitry) using unloaded Rink Amide MBHA resin (NovaBiochem, 0.25 mmol). The initial Gly residue was double coupled onto the resin. All additional amino acids, except for Arg, were single coupled. A standard double coupling protocol was used for Arg^8. The N-terminal Fmoc group was removed from the peptide and the resin dried (N_2). The resin was transferred to a manual peptide synthesis vessel with fritted bottom (N_2 used for agitation). The resin

was suspended in DMF (5 mL) and 3-mercaptopropionic acid (1 mmol) was manually coupled to the amino terminus using PyBOP/HOBt activation (1 mmol PyBOP, 0.1 mmol HOBt) and N_2 agitation until a Kaiser test of the resin showed that the reaction was complete (16-48 hr). The peptide was cleaved from the resin with 20 mL of degassed "reagent R" (90:5:3:2 TFA/thioanisole/ethanedithiol/anisole) for 3 hr at RT. The mixture was filtered and the filtrate concentrated *in vacuo*. The remaining residue was triturated with ether and the precipitate collected and dried (272.1 mg). The peptide was purified by RP-HPLC (Delta Pak C_{18}, RCM 2-50X10, 45 mL/min.,16->24% CH_3CN, 30 min.). The fractions containing the desired product were combined and lyophilized overnight, providing the peptide as a white powder (98 mg, 31%). Electrospray MS showed the expected molecular weight (M+H=1288).

Preparation 4. [(3-Mercaptopropanoyl)-Gln^1, dAla^6]-GnRH : [SEQUENCE ID NO: 10] $HSCH_2CH_2CONH$-Gln-His-Trp-Ser-Tyr-DAla-Leu-Arg-Pro-Gly-NH_2

The peptide was prepared, up to the Gln^1 residue, on an ABI 431A peptide synthesizer (Fmoc chemsitry) using unloaded Rink Amide MBHA resin (NovaBiochem, 0.25 mmol). The initial Gly residue was double coupled onto the resin. All additional amino acids, except for Arg, were single coupled. A standard double coupling protocol was used for Arg^8. The N-terminal Fmoc group was removed from the peptide (regular piperidine cycle on ABI) and the resin dried (N_2). The resin was transferred to a manual peptide synthesis vessel with fritted bottom (N_2 used for agitation). The resin was suspended in DMF (5 mL) and 3-mercaptopropionic acid (1 mmol) was manually coupled to the amino terminus using PyBOP/HOBt activation (1 mmol PyBOP, 0.1 mmol HOBt) and N_2 agitation until a Kaiser test of the resin showed that the reaction was complete (16-48 hr). The peptide was cleaved from the resin with 20 mL of degassed "reagent R" (90:5:3:2 TFA/thioanisole/ethanedithiol/anisole) for 3 hr at RT. The mixture was filtered and the filtrate concentrated *in vacuo*. The remaining residue was

triturated with ether and the precipitate collected and dried (232.0 mg). The peptide was purified by RP-HPLC (Delta Pak C_{18}, RCM 2-50X10, 45 mL/min.,15->30% CH_3CN, 30 min.). The fractions containing the desired product were combined and lyophilized overnight, providing the peptide as a white powder (112 mg, 35%). Electrospray MS showed the expected molecular weight (M+H=1303).

Preparation 5. DLys^6-DAla^{10}-GnRH : [SEQUENCE ID NO: 11] pGlu-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-DAla-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Lys-10-D-Ala-GnRH (positive ion, NBA matrix) Calc (m+1) = 1268.5; Found (m+1) = 1267.5.

Preparation 6. DLys^6-Pro^9-NHEt-GnRH: [SEQUENCE ID NO: 12] pGlu-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide was synthesized on or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (24 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide was precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups were removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF was removed in vacuo and the residue triturated with diethyl ether. The peptide was purified by preparative reverse phase HPLC. Characterization by FAB-MS (positive ion, NBA matrix) found (m + 1) = 1223.9.

Prepartion 7. DOrn^6-GnRH : [SEQUENCE ID NO: 13] pGlu-His-Trp-Ser-Tyr-DOrn-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Orn-GnRH (positive ion, NBA matrix) Calc (m+1) 1239.4; Found (m+1) 1239.5.

Preparation 8. 3-Indolylpropionyl-6-DLys-GnRH : 3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 3-indolylpropionyl-6-D-Lys-GnRH (positive ion, NBA matrix) Calc (m+1) 990.2; Found (m+1) 990.7.

Preparation 9. 3-Indolylpropionyl-6-DLys-9-Pro-NHEt-GnRH : 3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The 3-indolylpropionyl moiety is incorporated as N-formyl-3-indolepropionic acid. The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups are removed from the dry peptide by treatment with anhydrous HF

(0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

PREPARATION OF PSEUDOMONAS EXOTOXIN

Preparation 10. NLys PE38QQR

Plasmid PJH4 (Ref. Hwang. J. "Cell" (1987, 48; 129-136) contains the coding sequence for PE_{1-613}. Oligonucleotide directed mutagenesis as described in 15.51-15.73, Molecular Cloning, 2nd ed (1989) edited by Sambrook, Fritch & Maniatis (Cold Spring Harbor Press) has been used as a covenient way to make deletions/mutations in the PE molecule. An NdeI/Hind III double digest is carried out on PJH4 resulting in linearization of the construct and clipping of a 12 b_p segment which includes the ATG start codon of the PE coding sequence. Two complementary oligonucleotides are synthesized, annealed and ligated into the NDEI/Hind III splice site. The oligomers have the following nucleotide sequence: 1-5' TAT GCT GCA GGG TAC CAA GCT TAT GGC CGA AGA$^{3'}$ and II - 5' AGC TTC TTC GGC CAT AAG CTT GGT ACC CTG CAG CA3'. The modified PE insert has a sequence of MLQGTKLMAEE constructed at the N-terminus. This plasmid is designated PJH42.

The plasmid PJH42 is partially cut with Ava I. The linear form of DNA is isolated, completely digested with Hind III, and the resulting 5.1 Kb fragment isolated. S1 nuclease treatment is carried out to allow blunt end ligation and the plasmid is recircularized and designated PJH43. This results in a PE with deletion of amino acids 4-252.

A 553 bp Sal I/Bam Hl fragment of plasmid PJH43 is cloned into M13 mp19. An oligonucleotide, 50 nucleotides in length with the structure 5' GGC GTC GCC GCT GTC CGC CGG GCC GTT GGC CGC GCC GGC CTC GTC GTT GC3', is synthesized and annealed to the single stranded M13 vector to facilitate (loop out) mutagenesis generating

a deletion of amino acids 365-380 of the PE insert, resulting in the sequence:

....AGAANGPADSGDALL....
↑↑
364381

A 505 bp Sal I Bam Hl fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.7 Kb Sal l Bam Hl fragment of the plasmid PJH43. This new plasmid is designated PJH44.

A Bam Hl/EcoR l fragment of 460 nucleotides is excised from PJH44 and cloned into M13 mp19. This fragment contains the nucleotide sequence for three lysines that are mutated at the carboxy end of the coding sequence: lysines 590, 606 are mutated to glutamines and lysine 613 is mutated to an arginine. Oligo directed mutations are then carried out successively at each of the lysines with the following oligomers:

Lysines 590-5' GCT GAT CGC CTG TTC TTG GTC GGG GAT GCT GGA C 3'

Lysines 606-5' GTC CTC GCG CGG CGG TTG GCC GGG CTG GCT G 3'

Lysines 613-5' CGG TCG CGG CAG TTA ACG CAG GTC CTC GCG CGG 3'

The Bam Hl EcoR l fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.4 Kb Bam Hl/EcoR l fragment of the plasmid PJH44. The linearized plasmid is then recircularized, designated PJH45 and used for expression of the modified PE, identified as NLys PE38QQR, from a commercially available strain of E. coli, HB 101, available from Bethesda Research Laboratories. NLys PE38QQR is also known as Lys PE38M as described in published WO 93/15751 (Merck & Co., Inc.).

Preparation 10. Reduced NLys PE38QQR

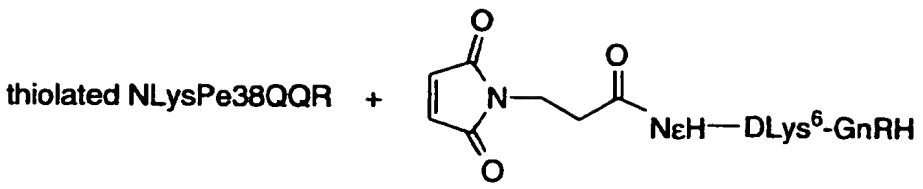
A solution of the toxin, NLys PE 38QQR, 10 ml containing 12.8 mg toxin, was adjusted to pH 8 by the addition of 175 mg of pH 8 buffer salts. EDTA disodium salt-dihydrate (372.24 mg) and 154.25 mg dithiothreitol were added and the mixture was shaken under dry nitrogen overnite at room temperature to complete the reduction of the cysteine 265, 287 disulfide bond in the toxin. The solution was transferred to a dialysis bag and dialyzed versus the buffer: 0.1 M phosphate (sparged with nitrogen) at room temperature for about eight hours. The reduced toxin solution was then dialyzed versus 0.01 M phosphate buffer overnite at room temperature with nitrogen sparging. At the end of the dialysis procedure the reaction contents from the dialysis bag were transferred to a sterile plastic centrifugation tube which was sampled for thiol content and the purified reduced toxin dithiol was retained for subsequent reaction.

Preparation 11. Photoinactivated PE holotoxin

Pseudomonas Exotoxin A (10 mg, 0.152 mmol) (Sigma Chemical Co.) was dissolved in 10 mL of water and to this was added 43 mg of 8-azidoadenosine (0.14 mmol). This did not completely dissolve and the small amount of precipitate was centrifuged and the supernatant was charged to a Pyrex photoreactor, cooled with ice water and irradiated with a 450 W Hanovia lamp for 8 min. The resultant solution was dialyzed for 17.5 hr against 4 L of PBS and then analyzed by TSK 2000 HPSEC which showed an absence of low molecular weight material (i.e. unreacted 8-azidoadenosine). The solution was also assayed for ADP ribosylating activity and found to have only 4% of the original level.

EXAMPLE 1

DLys^6-GnRH-NLys PE38QQR CONJUGATE (VIA SUCCINIMIDOPROPANOYL LINKER)



1) Preparation of (N_ε-maleimidopropanoyl)-DLys^6-GnRH : PyroGlu-His-Trp-Ser-Tyr-(N_ε-maleimidopropanoyl)-D-Lys-Leu-Arg-Pro-Gly-NH_2:

DLys^6-GnRH (10 mmol, 12.5 mg) was dissolved in N,N-dimethylformamide (0.5 mL/mg) and DIEA (50 mmol, 9 µL) added. The mixture was stirred briefly (RT) and β-maleimidopropionic acid N-hydroxysuccinimide ester (MPS; 20 mmol, 5.2 mg) was introduced in one portion. After 30 min reaction time, 10 µL TFA was added to the reaction mixture and the solvent removed in vacuo. The peptide was purified by reverse phase HPLC (Waters PrepPak® 25 x 10™ Delta-Pak™ C_{18}; 10 mL/min; 10-25 % B, 0-30 min.; then 25% B, 30-35 min; λ=230 nm). FAB-MS (positive ion, NBA matrix) Calc. M+1: 1405.56; Found M+1=1405.6.

2) Conjugation of N_ε-maleimidopropanoyl)-DLys^6-GnRH to NLys-PE38QQR

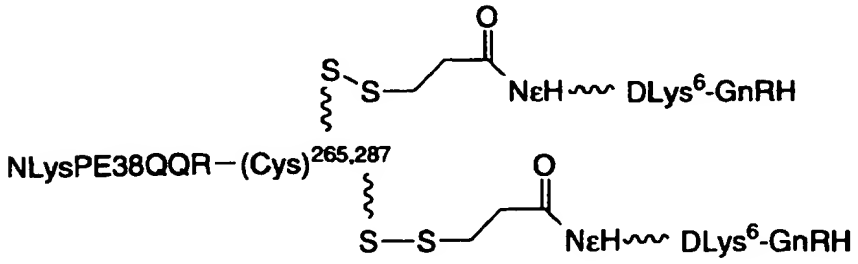
To a sterile 15 mL polyethylene centrifuge tube with septum was added NLys-PE38QQR (0.22 µmol, 30. mL, 2.8 mg/mL) in PBS. The pH of the solution was adjusted to 10.8 by the addition of 350 µL of 1.0 M, pH 11.0 borate buffer. Dithiothreitol (11.0 µmol, 1.7 mg) and EDTA-2Na (22.1 µmol, 8.2 mg) were added and the protein mixture vortexed until all solids were in solution. N-Acetylhomocysteine thiolactone (22.1 µmol, 3.5 mg) was introduced in one portion and the solution degassed and purged with nitrogen (degas/purge repeated 5X). The mixture was aged in an nitrogen box at RT for 6.5 h, then charged to Spectropor 2 dialysis tubing and dialyzed (RT) as follows: vs. 1) 4L degassed, nitrogen sparged, 0.1 M, pH 8.0 phosphate buffer which contained 10 mg EDTA-2Na, and 0.25 mg DTT (16 h); 2) 4L degassed,

nitrogen sparged, 0.01 M, pH 8.0 phosphate buffer which contained 10 mg EDTA-2Na, and 0.25 mg DTT (6 h). The thiolated exotoxin was then transferred to a sterile 15 mL polyethylene centrifuge tube (3.90 mL). An Ellman assay on 325 µL of this material indicated that a total of 0.350 µmol SH was present. To the remaining thiolated material (0.321 mmol SH, 3.57 mL solution) was added (Nε-maleimidopropanoyl)-DLys^6-GnRH (1.60 µmol, 2.25 mg). The reaction was then vortexed briefly and aged in an N2 box (RT, 1h). The toxin mixture was charged to Spectropor 2 dialysis tubing and dialyzed (4°C) as follows vs. 1) 4L, 0.01 M, pH7.0 phosphate buffer (18 h); 2) 4 L, 0.01 M, pH 7.0 phosphate buffer (46 h); and 3) 4 L deionized water (7h). The conjugate was centrifuged to pellet any unsuspended material and passed through a sterile filter (Millipore 0.22 µM, Millex®-GV). This provided the title conjugate, which had HPLC characteristics that were distinct from unconjugated NLys-PE38QQR.

Lys-PE38M RP-HPLC (250 mm x 4.6 mm Vydac C4; 1.5 mL/min.; 36-41% B, 0-30 min.; λ = 215 nm): RT = 18.16 min.

EXAMPLE 2

DLys^6-GnRH-NLys PE38QQR CONJUGATE (VIA DITHIOPROPANOYL LINKER)



DLys^6-GnRH (15 mg, 12 µmol) was dissolved in dry DMF (3 mL) and DIEA (2 µL, 12 µmol) was added. SPDP (N-succinimidyl-3-[2-pyridyldithio]propionate, 4.1 mg, 13 µmol) was added in one portion

and the reaction stirred overnight (18 h) at room temperature under an atmosphere of nitrogen. The reaction was concentrated *in vacuo* and the residue purified by HPLC (high performance liquid chromatography; Waters Delta Pak C_{18}, RCM 25x10, 10 mL/min, 15->45% CH_3CN gradient over 30 min). The fractions containing the desired product were combined and lyophilized, affording [DLys^6-N-(3-dithiopyridyl)propanoyl]-GnRH as a white powder.

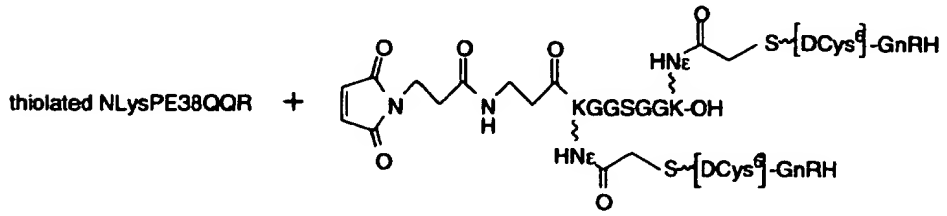
Electrospray MS [M+H]= 1450.3

[DLys^6-N-(3-dithiopyridyl)propanoyl]-GnRH (1.2 mg, 0.86 µmol) was added to reduced NLys PE38QQR (5 mL, 0.285 µmol). the reaction was vortexed and agitated overnight (4 °C to room temperature) for 15 h. The reaction mixture was transferred to dialysis tubing and dialyzed *vs*: 4 L, 0.01 M pH 8.0 phosphate buffer sparged with nitrogen, RT, 7 h; and 18 L, 0.9% aq. NaCl, RT, 20 h. The solution was filtered (0.22 µm Millex GV sterile filter). HPLC analysis vs. a known standard of NLys PE38QQR revealed the protein concentration of the resultant conjugate to be 1.2 mg/mL.

HPLC of conjugate: (Vydac, C_4, 4.7x250 mm, 1.5 mL/min, 35->45% CH_3CN, 20 minutes gradient); retention time=16.27 min

EXAMPLE 3

[DCys^6]GNRH-KGGSGGK-NLYS PE38QQR CONJUGATE



1) Preparation of Fmoc-β-Ala-Lys-Gly-Gly-Ser-Gly-Gly-Lys-OH (linear scaffold):

The linear scaffold was prepared on an ABI 431A peptide synthesizer (Fmoc chemistry) using pre-loaded Fmoc-Lys-WANG resin and single amino acid couplings. The peptide was cleaved from the resin TFA/thioanisole/ethenedithiol/anisole (90:5:3:2) and purified by gradient RP-HPLC. Electrospray MS (M+H = 882.0)

2) Preparation of Fmoc-β-Ala-K(N_εHBrAc)GGSGGK(N_εHBrAc)OH (bis-bromoacetylated linear scaffold):

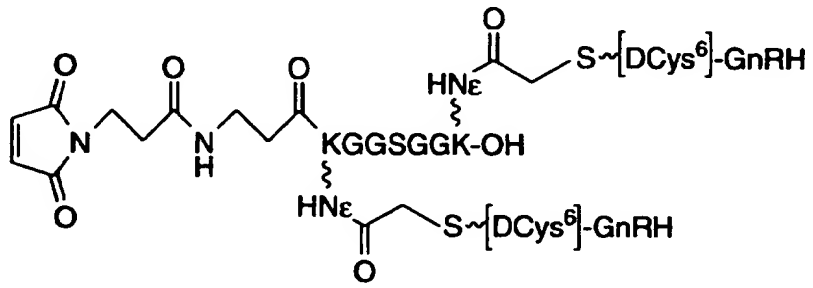
Bromoacetic anhydride was prepared by reaction of bromoacetic acid (31.5 mg, 0.226 mmol, 3.96 eq.) and DCC (23.4 mg, 0.113 mmol, 1.98 eq.) in dry (Aldrich Sure-Seal) dichloromethane (2 mL) at room temperature for 1 h. This mixture was filtered (sintered funnel, N_2 pressure) directly into a solution of the linear scaffold peptide from step 1 (50.0 mg, 0.057 mmol) that was dissolved in dry (4 Å sieves) degassed DMF (5 mL). The reaction was allowed to proceed at room temperature under N_2. Analysis of the reaction by RP-HPLC (Vydac C_{18}, 4.7X250 mm, 1.5 mL/min, 10->60% CH_3CN, 20 min) after 30 min indicated that all starting material had been consumed (ret. time for linear scaffold =13.28 min) and a new product peak was observed (R.T.=16.48 min). After 45 min total reaction time, the mixture was concentrated *in vacuo* and the product purified by RP-HPLC (DeltaPak C_{18}, RCM 25X10, 10 ml/min, 25->55% CH_3CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the bis-bromoacetylated linear scaffold as a white powder (31.2 mg, .027 mmol, 49%).

3) Preparation of Fmoc-β-Ala-K(N_εHCOCH$_2$S~[DCys^6]-GnRH)-GGSGGK(N_εHCOCH$_2$S~[DCys^6]-GnRH)-OH (scaffold supported [DCys^6]-GnRH):

The bis-bromoacetylated linear scaffold from step 2 (31.2 mg, 0.027 mmol) was dissolved in degassed pH 8.0, 0.10 M phosphate buffer (5 mL) and acetonitrile (250 µL) was added. A pH meter indicated that the solution had a pH of 8.1. Next, [DCys^6]-GnRH (68.1 mg, 0.055 mmol, 2.04 eq.) was dissolved in water (2 mL) and a few drops of

acetonitrile were added to clear the solution. The solution of [DCys^6]-GnRH was added dropwise (slow addition over 35 to 40 minutes) to the bis-bromoacetylated scaffold while maintaining the pH of the reaction mixture between pH 7.8 to 8.1 with the addition of dilute ammonium hydroxide. The final pH of the reaction mixture was 8.0. The reaction mixture was stirred at room temperature for 10 min then sonicated for 20 min after which time the mixture became cloudy. Stirring was again maintained at room temperature for a total of 2 h reaction time. RP-HPLC analysis of the reaction mixture (Vydac C_{18}, 4.7X250 mm, 1.5 mL/min, 10 to 60% CH_3CN, 20 min) indicated that all starting material was consumed and a new product peak was observed (R.T.=14.34. min). The reaction mixture was concentrated *in vacuo* and the remaining residue taken up in 6M guanidine hydrochloride and purified by RP-HPLC (DeltaPak C_{18}, RCM 25X10, 10 ml/min, 20 to 50% CH_3CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the scaffold supported [DCys^6]-GnRH as a white powder (51 mg, .015 mmol, 49%). Electrospray MS (M+H) = 3419

4) Preparation of maleimidopropanoyl-β-Ala-K(N_εHCOCH$_2$S~[DCys^6]-GnRH)GGSGGK(N_εHCOCH$_2$S~[DCys^6]-GnRH)-OH (maleimidated scaffold supported [DCys^6]-GnRH):



(a) Cleavage of Fmoc. The scaffold supported [DCys^6]-GnRH from step 3 (11.4 mg, 0.0033 mmol) was dissolved in 20% piperidine (Aldrich) in dry DMF (4Å sieves) (3 mL). The solution was

stirred at room temperature for 45 min then concentrated *in vacuo*. The residue was taken up into ~15% aq. CH_3CN and an aliquot was removed for RP-HPLC analysis. HPLC analysis showed approximately a 83:17 ratio of product to starting material (Vydac C_{18}, 4.7X250 mm, 1.5 mL/min, 10->60% CH_3CN, 20 min; RT=11.28 min deprotected peptide product, RT=14.72 min Fmoc-protected peptide starting material). The remaining material was lyophilized overnight affording the deprotected peptide as a white fluffy solid which was used directly in the maleimidation.

(b) Maleimidation of deprotected scaffold supported [DCys^6]-GnRH. The deprotected peptide (0.0033 mmol) was dissolved in dry (4Å sieves) degassed DMF (2.5 mL) and DIEA (10 µL) added. The mixture was stirred at room temperature and MPS (1.8 mg) was added in one portion. After 30 min an aliquot was removed for RP-HPLC analysis. HPLC analysis showed the appearance of two new products and a small amount of starting material (Vydac C_{18}, 4.7X250 mm, 1.5 mL/min, 10 to 60% CH_3CN, 20 min; RT=11.28 min deprotected peptide starting material, RT=12.13 min maleimidated peptide product). At T=45 min reaction time, the reaction mixture was quenched by the addition of TFA (10 µL) and concentrated *in vacuo*. The remaining residue was taken up into 10% aq. CH_3CN (0.1% TFA) and purified by RP-HPLC (DeltaPak C_{18}, RCM 25X10, 10 ml/min, 20 to 40% CH_3CN, 30 min). The fractions containing the desired material were combined and lyophilized overnight, providing the maleimidated scaffold supported [DCys^6]-GnRH as a white powder (6.9 mg, .0021 mmol, 62%). Electrospray MS showed that the product had the desired molecular weight (M+H=3348).

5) Thiolation of carrier protein NLys PE38QQR:

The protein (NLys PE38QQR, 1.28 mg/mL, 20 mL, 25.6 mg, 0.66 µmol) was placed in a 50 mL plastic sterile centrifuge tube and the pH 11 borate buffer salts (832 mg, 41.6 mg/mL affords a 0.1 M solution of pH 11 borate buffer) were added and the mixture was capped and vortexed until all solids were in solution (5 min). EDTA (100 mg)

and DTT (10 mg, 0.065 mmol) were added to the reaction mixture and the solution again vortexed until all solids were dissolved. The tube was transferred into an N_2-filled box and the cap replaced by a rubber septum.. The tube was evacuated briefly and purged with N_2 (repeated 5X). N-acetylhomocysteine thiolactone (100 mg, 0.629 mmol) was added in one portion and the mixture vortexed until all solids were in solution and the tube re-evacuated and purged with N_2 (repeated 5X). The tube was capped and allowed to age in the N_2-filled box overnight (20 h) at room temperature. The reaction mixture was transferred to a dialysis bag and dialyzed against (a) 4L, 0.1 M pH 8.0 phosphate buffer with N_2 sparging (19 h); (b) 4L, 0.01 M pH 8.0 phosphate buffer containing 100 mg EDTA with N_2 sparging (8 h); and (c) 4L, 0.01 M pH 8.0 phosphate buffer containing 100 mg EDTA with N_2 sparging (20 h). The thiolated protein was transferred (in the N_2 box) to a 50 mL plastic centrifuge tube, approximately 24 mL volume. A 200 µL aliquot was removed for an Ellman's analysis (OD_{412}=0.155, 1.5 mL total volume) which revealed that the protein solution had a thiol titre of 0.083 µmol SH/mL solution.

6) Conjugation of Thiolated NLys PE38QQR and Maleimidated Scaffold Supported [pCys^6]-GnRH:

The maleimidated peptide of step 4 (5.0 mg 1.49 µmol) was dissolved in water (0.1% TFA) and placed in a sterile 50 mL plastic centrifuge tube and lyophilized overnight. The thiolated protein of step 5 (0.083 µmol SH/mL protein, 16.9 mL, 1.40 µmol) was added to the lyophilized peptide and the tube capped and vortexed briefly. The tube was sealed with parafilm and placed on a Clay-Adams nutator and tumbled overnight at 4 °C (17 h). The reaction mixture was transferred to a dialysis bag (Spectropor 2) and dialyzed (4 °C) *vs.* (a) 4L, 0.01 M pH 7.0 phosphate buffer (8 h); (b) 4L, pH 7.0 Dulbecco's phosphate buffered saline (PBS) (72 h), and (c) 4L, pH 7.0 Dulbecco's phosphate buffered saline (PBS) (24 h). There was no visible precipitate present. The conjugate was sterile filtered (sterile 50 mL Corning cup filter, 0.22 µM), providing approximately 15 mL of product. The conjugation product was

characterized by CZE and SDS-PAGE gel electrophoresis. The concentration of the product was established (CZE) to be 1.1 mg/mL *vs.* known standards of TP40 (a chimeric protein containing transforming growth factor alpha at the N-terminus and a derivative of a 40 kDa segment (PE40deltacys) of Pseudomonas exotoxin) and NLys PE38QQR.

EXAMPLE 4
PHOTOINACTIVATION OF THE CONJUGATE OF EXAMPLE 3

The conjugate of Example 1 (0.5 mL) was added to 9.5 mL of phosphate buffered saline (PBS) containing 4.16 mg of 8-azidoadenosine affording a solution which is .29 mM in conjugate and 1.35 mM in azidoadenosine. This solution was charged to a Pyrex photoreactor with a cooling jacket through which ice water was pumped. It was then irradiated using a 450 W Hanovia lamp at a distance of 6 inches for 6 min. The solution was then dialyzed vs. 4 L of PBS 16 hr affording solution which had no low molecular weight materials as indicated by TSK 2000 HPSEC. The ADP ribosylating activity of this material was only 20% of the original as indicated by a wheat germ assay.

EXAMPLE 5
[DCys^6]GNRH-KGGSGGK-PHOTOINACTIVATED PSEUDOMONAS EXOTOXIN (HOLOTOXIN) CONJUGATE

1) Thiolation of Photoinactivated PE holotoxin.

Borate buffer salt (43 mg [equal to 12.7 mmol/ mL of final solution] was added to 1 mL of water to which was added 10 mg of EDTA and 2.2 mg DTT. This thiolation medium was added to 9.5 mL of the photoinactivated PE toxin solution. N-acetylhomocysteine thiolactone (11.3 mg) was added, the solution degassed and the air replaced with nitrogen and aged in a nitrogen box for 16 hr. It was then dialyzed against 4 L of PBS for 7.5 hr and a fresh 4 L of PBS for 65 hr at 4° C .The dialysis solutions were sparged with nitrogen throughout. An Ellman assay indicated 65 nanomoles of thiol / mL.

2) Conjugation of thiolated photoinactivated Pseudomonas exotoxin and maleimidated scaffold supported [DCys^6]GnRH.

The maleimidated scaffold supported [DCys^6]GnRH of Example 4, step 4 (1.88 mg, 450 nanomoles) was dissolved in 100 µL of water and 95 µL of this was added to 4 mL of the thiolated, photoinactivated PE holotoxin prepared in step 2. This was degassed and aged for 16 hr. At this time TSK 2000 HPSEC shows considerable low m.w. material remains. The solution was then dialyzed for 7 hr against 4L of PBS and then for 178 hr against a fresh 4 L of PBS at 4°C. An HPSEC assay shows no small molecules remain. An amino acid analysis indicates that there are 90 µg of scaffold / mL (β-alanine content) and 517 µg of PE toxin / mL.

EXAMPLE 6
Vaccine Preparation and Efficacy Screening

The vaccines were prepared by combining the peptide solutions with 0.9% sodium chloride injection USP (Baxter, Lot C255075) and Incomplete Freund's Adjuvant (Sigma, F 5506, Lot 062H-8802) in the proportions listed below in a 20 cc, glass, luer-lok syringe (Popper & Sons). Homogenization was achieved by passing the mixture between two 20 cc glass syringes through a 20 gauge, double hubbed, homogenization needle (Popper & Sons) until stiff.

Vaccine No.	Antigen (Ex.3 in mg/ml)*	Saline	IFA	Final Ag Conc.
1	3 ml (1.17)	4 ml	7 ml	250 ug/ml
2	6 ml (0.117)	1 ml	7 ml	50 ug/ml
3	0.6 ml (0.117)	6.4 ml	7 ml	5 ug/ml
4	6 ml (0.00117)	1 ml	7 ml	0.5 ug/ml

* the numbers in parenthesis represent the concentrations of the conjugate of Example 3.

In addition, an alum adjuvanted vaccine was also prepared by mixing 6 ml of immunogconjugate of Example 3 (0.117 mg/ml in saline), 1 ml saline and 7 ml Alhydrogel 1.3% (Superfos Biosector a/s, Batch 1983) to yield antigen final concentration of 50 ug/ml (vaccine No. 5).

Vaccination:

Twenty seven 10 week-old, castrated male pigs were divided into six groups: one group of 5 animals for each of the above vaccine preparations plus a group of 2 control animals. Pretreatment blood samples (10 ml) were collected by jugular venipuncture and each pig was vaccinated with two ml of freshly prepared vaccine (1 ml intramuscularly on each side of the neck). Four weeks later, blood (10 ml) was again collected from all animals and they were revaccinated with freshly prepared vaccine as previously described. Two weeks later, all animals were bled three times at approximately 1 hour intervals. All blood samples were centrifuged following collection and serum was frozen at -20 C until all of the bleeding were completed so that all serum could be assayed in the same antibody titer and LH analyses.

Antibody Titer Analysis:

PBS-BSA was prepared by dissolving one packet of BupH modified Dulbecco's phosphate buffered saline mix (Pierce, No. 28374, Lot 920521084) in 400 ml of deionized water and adding 2 gm of bovine albumin fraction V (Gibco No. 810-1018IL, Lot 76P9623) and 5 ml of a 1% w/v thimerosal solution (Sigma, T-5125, Lot 23H0526). Once the BSA was dissolved, deionized water was added to a final volume of 500 ml.

Dextran-coated charcoal suspension was prepared by washing 2.5 g of charcoal (Sigma, C-5385, Lot 102H0336)with deionized water multiple times to remove the fines. PBS was prepared by dissolving two packet of BupH Modified Dulbecco's Phosphate Buffered Saline mix (Pierce, No. 28374, Lot 920521084) in 1000 ml of deionized water. 0.25g of dextran, 70,000 mw (Sigma, D1390, Lot 122H0349) was dissolved in 500 ml of PBS. The washed charcoal was added to this

solution. 10 ml of 1% w/v thimerosal solution (Sigma, T-5125, Lot 23H0526) plus an additional 500 ml of PBS were added and the charcoal suspension was stirred for 3 days at 4 C.

All serum samples were thawed. A 100 fold dilution of serum was prepared by adding 5 ul of serum to 495 ul of PBS-BSA. A 1000 fold dilution of serum was prepared by adding 50 ul of the 100 fold dilution to 450 ul of PBS-BSA. 10,000 and 100,000 fold dilutions were prepared similarly. Fifty ul aliquots of all serum dilutions of were added to duplicate 12x75 borosilicate glass tubes (Fisher) (two tubes per dilution). To each tube were added 50 ul of PBS-BSA containing 400,000 cpm/ml of ^{125}I labeled-GnRH (NEN, NEX-163, Lot CF91640). Tubes were incubated overnight at 4 C. Then 100 ul of dextran coated charcoal suspension was added and tubes were mixed at room temperature for 15 minutes. Tubes were then placed in a Sorval RC-3B centrifuge and spun at 2500 rpm in an H6000 A rotor. A 100 ul aliquot of the supernatant was collected and the radioactivity was quantified in a Packard AutoGamma 800 gamma counter. Results were expressed as percentage binding of the total input radioactivity (determined by adding 50 ul of the ^{125}I labeled GnRH solution to 150 ul of PBS-BSA, centrifuging and counting 100 ul).

The antibody titer results were as follows:
Values are expresses as the average percentage of input ^{125}I-labeled peptide which was bound at a 1/1000 dilution of serum except for pretreatment which is neat serum.

Vaccine No.	Pretreatment	4 Weeks	6 Weeks
1	1.4%	15.0%	52.7%
2	1.2%	8.2%	39.2%
3	1.4%	5.9%	37.1%
4	1.0%	4.1%	12.3%
5	0.9%	0.5%	8.0%
Control	0.5%	-0.3%	-0.4%

Serum LH Determination

Serum samples were submitted to the USDA lab at Athens, GA under the supervision of Dr. George Rampacek for serum LH quantitation by radioimmunoassay. The assay is a standard radioimmunoassay using ^{125}I labelled porcine LH and anti-bovine LH antisera which recognize porcine LH. This assay is described by R. Kraeling et al in *J. Anim. Sci.* (1982) 54:1212. The results below are the means for each group. Week 6 is the pooled mean for the three bleedings. The results are presented in ng of LH/ml. 0.15 ng LH/ml of serum is the lower limit of detection for the assay so all values which fell below the level of detection were assigned the value of 0.15 ng/ml.

Vaccine No.	Pretreatment	4 Weeks	6 Weeks
1	1.85	0.15	0.15
2	1.46	0.44	0.15
3	1.41	0.52	0.15
4	0.87	0.85	0.16
5	0.82	0.72	0.24
Control	1.50	1.22	0.96

EXAMPLE 8

Vaccine formulation with STP

1. Preparation of STP (5% Squalane: 0.2% Tween 80: 2.5 % Pluronic 121 in phosphate buffered saline [PBS]) :

Squalane (500 mg), Pluronic 121 (250 mg; a block copolymer of polyethylene oxide and polypropylene oxide (BASF corp.) and Tween 80 (20 mg) are weighed into a 15 mL Dounce tissue homogenizer tube. This is then covered with 9.25 mL of PBS (pH 7.4) and the resultant mixture homogenized with about ten strokes . The solution is then transferred to a vial and a small magnetic stir bar added.

About 3 mL of this mixture is transferred into cylinder of an Avestin Emulsiflex® microfluidizer. and the exit tube of the Emulsiflex is positioned so that it is submerged below the surface of the remaining liquid in the vial The vial is cooled in an ice bath, and twenty passes of the Emulsiflex are effected while the liquid is magnetically stirred. In this manner 9.5 mL of STP are obtained.

2. Formulation of the conjugate of Example 3 in STP 130 µL of a solution of the conjugate of Example 3 (1µg /µL of total immunogen = 130 µg total) is added with magnetic stirring to 6.4 mL of the above STP emulsion and the resultant mixture subjected to 10 passes in the Emulsiflex® unit using the same procedure as in the preparation of STP. This affords about 6 mL of the conjugate formulated in STP at a concentration of 20 µg/ mL. The same product can be obtained by adding the requisite amount of the conjugate (in PBS) to the squalane:Tween 80:Pluronic 121 mixture in the Dounce homogenizer in Procedure 1, and subjecting the four part mixture to 20 passes in the microfluidizer.

3. Formulation of the conjugate of Example 3 in STP with Dehydroepiandrosterone (DHEA): : 130 µL of solution of the conjugate of Example 1 (1µg /µL of total immunogen = 130 µg total) is added with magnetic stirring to 6.2 mL of the STP emulsion prepared in section 1. A solution of DHEA (10 µg/µL) in ethanol is prepared and 195 µL of this solution (1.95 mg) is added to the stirred conjugate solution in STP. This mixture is then subjected to 10 passes through the Emulsiflex instrument, to afford about 6 mL of conjugate in STP with DHEA.

The general procedure of Steps 2 and 3 was followed to provide [DLys^6]GnRH-NLysPE38QQR vaccines in STP and STP + DHEA.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hickey, Gerard J.
Mohn, Kenneth L.

(ii) TITLE OF INVENTION: PSEUDOMONAS EXOTOXIN AS IMMUNOGENIC CARRIER IN SYNTHETIC CONJUGATE VACCINES

(iii) NUMBER OF SEQUENCES: 13

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(E) COUNTRY: USA
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Yang, Mollie M.
(B) REGISTRATION NUMBER: 32,718
(C) REFERENCE/DOCKET NUMBER: 19445

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 908-594-6343
(B) TELEFAX: 908-594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Xaa His Trp Ser Trp Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa His Trp Ser Trp Xaa Xaa Arg Xaa Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 356 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Leu Gln Gly Thr Lys Leu Met Ala Glu Glu Gly Gly Ser Leu Ala
1 5 10 15

Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe Thr
20 25 30

Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr
35 40 45

Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp
50 55 60

Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser
65 70 75 80

Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg
85 90 95

Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln
100 105 110

Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser
115 120 125

Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu
130 135 140

Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn Trp
145 150 155 160

Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg Gly
165 170 175

Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser
180 185 190

Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile
195 200 205

Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr
210 215 220

Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala
225 230 235 240

Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg
245 250 255

Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg
260 265 270

Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro
275 280 285

Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala
290 295 300

Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn
305 310 315 320

Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Gln Glu Gln Ala
325 330 335

Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Gln Pro Pro Arg
340 345 350

Glu Asp Leu Arg
355

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Gly Gly Ser Gly Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Gly Gly Thr Gly Xaa Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa His Trp Ser Tyr Xaa Leu Arg Pro Gly
1 5 10

WHAT IS CLAIMED IS:

1. A method for eliciting anti-GnRH antibodies in an animal which comprises administering to said animal an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereof, in an amount effective to elicit anti-GnRH antibodies.

2. A method for sterilizing an animal comprising administering to said animal an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereof, in an amount effective to achieve sterilization.

3. A method of Claim 1 wherein said immunogenic carrier system comprises a GnRH-Pseudomonas conjugate.

4. A method of Claim 2 wherein said immunogenic carrier system comprises a GnRH-Pseudomonas conjugate.

5. A vaccine composition comprising an immunogenic carrier system comprising a GnRH associated with Pseudomonas exotoxin or a variant thereof, in an amount effective to elicit anti-GnRH antibodies, and a vehicle capable of augmenting the immunogenicity of said immunogenic carrier system.

6. A method for sterilizing an animal comprising administering to said animal a vaccine composition of Claim 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17008

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/104, 51/10; C07K 16/42
US CL :424/181.1, 195.11, 197.11; 530/388.24, 398
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/181.1, 195.11, 197.11; 530/388.24, 398

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, HCAPLUS, BIOSIS, EMBASE
search terms: antibodies, GnRH, carrier system, Pseudomonas, immunogenic, sterilizing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,378,688 (NETT ET AL.) 03 January 1995, see entire document.	2 and 4
X, P	US, A, 5,488,036 (NETT ET AL.) 30 January 1996, see entire document.	2 and 4
X, P	US, A, 5,492,893 (NETT ET AL.) 20 February 1996, see entire document.	2 and 4
Y	SAD et al. Bypass of Carrier-Induced Epitope-Specific Suppression Using a T-Helper Epitope. Immunology. 1992. Vol. 76. pages 599-603, see entire document.	1, 3, 5, and 6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 JANUARY 1997	Date of mailing of the international search report 12 FEB 1997
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